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COW COLOSTRUM ACUTE PHASE PROTEINS (SAA, HP) AND CYTOKINES (IL-1β, IL-6): ASSOCIATIONS WITH INFLAMMATORY RESPONSE IN NEONATAL CALVES

VEISE TERNESPIIMA ÄGEDA JÄRGU VALKUDE (SAA, HP) JA TSÜTOKIINIDE (IL-1β, IL-6) SISALDUSE MÕJU VASTSÜNDINUD VASIKA PÕLETIKUVASTUSELE

Final Thesis
Curriculum in Veterinary Medicine

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Colostrum is known to be vital for a newborn calf. It confers passive immunity and some of its components are suggested to be important for the development of calf's immature immune system. Aim of the study was to investigate possible associations of colostral acute phase proteins (SAA and Hp) and cytokines (IL-1β and IL-6) concentrations to the same variables in calves' serum in a research period from birth until three weeks of life after one-time ingestion of colostrum after birth.

This study used a research material gathered for a large-scale study in 2015. The study population comprised of 144 female calves from a large dairy farm in Central-Estonia. Colostrum (n = 144) and calves' serum samples (n = 383) were used to measure the studied concentrations, which were statistically evaluated after logarithmic transformation by using linear or Tobit regression models.

The studied colostrum concentrations (median, min-max) measured were for SAA 52.2 mg/l (7.5–277.0), Hp 174.6 mg/l (103.7–479.5), IL-1β 115.1 ng/l (15.6–5445.1) and IL-6 43.5 ng/l (10.2–501.4). Colostrum IL-1β and IL-6 concentrations were observed to have a positive association with the same concentration variables in calves' serum during the first week of life ($p < 0.001$). These results suggest that colostrum cytokines IL-1β and IL-6 may be transferred to calf by passive transfer and/or stimulate calf's own cytokine production. Also, colostrum and calves' serum IL-6 concentrations were observed to have a positive association with each other during the second ($p < 0.001$) and third week of life ($p = 0.001$). The cause for this long-term association is unknown and needs further research. Furthermore, colostrum IL-6 concentration had a positive association to calves' serum Hp concentration during the first week of life ($p < 0.001$), which may indicate that colostrum cytokines have effect to the calves' immune response development and maturation by inducing the calf's liver to produce acute phase proteins.

Keywords: neonatal calves, colostrum, cytokine, acute phase protein, immune system

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Juhendaja(d): Toomas Orro ja Kristel Peetsalu
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<tr><td colspan="4">Vastsündinud vasika jaoks on ternespiim väga oluline, sest sisaldab erinevaid komponente, mis on vajalikud passiivse immuunsuse omandamiseks ja immuunsüsteemi väljakujunemise jaoks. Uuringu eesmärk oli leida ternespiimas sisalduvate ägeda järgu valkude seerumi amüliod A (SAA) ja haptoglobiini (Hp) ning tsütokiinide interleukiin (IL)-1β ja IL-6 kontsentratsioonide võimalik seos vasika seerumi samade parameetritega. Uuringuperiood kestis vasikate sünnist kuni kolmanda elunädalani. Vasikaid joodeti sel perioodil ternespiimaga üks kord. Uuringumaterjal, mis moodustab ühe osa suuremast uurimisprojektist, koguti 2015. aastal Kesk-Eesti suurest piimafarmist. Uurimispopulatsioon koosnes 144 lehmvasikast. Ägeda järgu valkude ja tsütokiinide kontsentratsioonide sisalduse määramiseks kasutati ternespiimaproove (n = 144) ja vasikate seerumiproove (n = 383). Tulemusi hinnati statistiliselt peale logaritmilist teisendamist ning kasutati lineaarset või Tobiti regressioonimudelit.
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<tr><td colspan="4">Märksõnad: vastsündinud vasikas, ternespiim, tsütokiin, ägeda järgu valk, immuun süsteem</td></tr>
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LIST OF ABBREVIATIONS

APP	acute phase protein
APR	acute phase response
$CD4^+$	T helper cell (lymphocyte)
$CD8^+$	T cytotoxic cell (lymphocyte)
Hp	haptoglobin
IFN-γ	interferon-gamma
Ig	immunoglobulin (e.g., IgG, IgA, IgM, IgE)
IL-1	interleukin-1
IL-1ra	interleukin-1 receptor antagonist
IL-1α	interleukin-1-alpha
IL-1β	interleukin-1-beta
IL-2/-4/-8/-10/-12/-13/-18	interleukin-2/-4/-8/-10/-12/-13/-18
IL-6	interleukin-6
LPS	lipopolysaccharide (the major component of the outer membrane of Gram-negative bacteria)
M-SAA3	mammary-associated serum amyloid A3
MHC I/II	major histocompatibility complex I/II
mRNA	messenger RNA
MUC3	mucin 3 gene; small intestine membrane associated mucin
O_2^-	superoxide anion (radical)
PAMPs	pathogen-associated molecular patterns
PRRs	pattern recognition receptors
rpm	revolutions per minute
SAA	serum amyloid A
TGF-β	transforming growth factor-beta
Th1	T helper cell type 1
Th2	T helper cell type 2
TNF-α	tumor necrosis factor-alpha

INTRODUCTION

Colostrum is known to provide a complete nutrition for a newborn calf, but in addition it confers passive immunity, which is known to be vital for the calf's adaptation and survival (Weaver *et al.*, 2000; Woolums, 2012). During pregnancy and at the time of parturition the hormones have immunosuppressive effect to the newborn calf's immune system (Chase *et al*., 2008). The passive transfer of maternal immunoglobulins through colostrum aids the newborn calf's immune defence during the period when the immune system is still immature. In addition, it is suggested that colostrum contains other important active components that have physiological functions, which develop and mature the calf's immature immune system (Nissen *et al*., 2017).

Bovine colostrum is known to contain immune components like cytokines, which are found in high concentrations in colostrum at the time of parturition (Hagiwara *et al*., 2000; Yamanaka *et al.*, 2003). Cytokines are known to have various effects and functions to different cells in the organism, although lot is still unknown. They have important role in the functions of immune system (Kaiser, 2020). They induce innate immune system's acute phase response (APR), which is the first defence mechanism against pathogens (Hiss *et al*., 2004). Proinflammatory cytokines trigger liver to produce acute phase proteins (APPs) like serum amyloid A (SAA) and haptoglobin (Hp), which have role in protecting and defending the animal against the pathogens encountered (Tizard, 2004: 43–44). Colostral cytokines' role in newborn calves isn't entirely understood. Their passive transfer and effect to APPs concentrations in the serum of post-natal calves is somewhat unclear (Orro *et al*., 2006; Orro *et al*., 2008; Hiss-Pesch *et al*., 2011), because colostrum contains APPs also (Thomas *et al*., 2016). Colostral cytokines are suggested to contribute to the maturation and development of newborn calf's immune system (Hagiwara *et al*., 2000; Hagiwara *et al*., 2001; Yamanaka *et al*., 2003; Gomes *et al*., 2014).

Interest lies especially in the possible associations between colostrum cytokines and the post-natal calves' serum acute phase protein concentrations, while having possible effect to newborn calf's immune response and development.

1. LITERATURE REVIEW

1.1. Newborn calf and adaptation to extrauterine life

At the time of parturition enormous physiological changes occur in the calf's life making it as one of the most challenging periods (Kirovski, 2015). This neonatal period is one of the most critical, because of high morbidity and mortality rates of newborn calves (Ignătescu *et al.*, 2018). This adaptive period is a transition phase during which all organ functions must adapt to the new environment (Nagyová *et al.*, 2017). It means that the newborn calf has to take over the vital functions, which the mother took care earlier (Ignătescu *et al.*, 2018). During neonatal period calf's thermoregulatory, cardiorespiratory, endocrine and metabolic systems go through marked changes (Blum and Hammon, 2000; Kirovski, 2015). In addition, calf's immune system starts its maturation (Kirovski, 2015). Often the high morbidity and mortality in young calves are related to their inability to adapt to the extrauterine life and take over the vital functions to survive (Ignătescu *et al.*, 2018).

Newborn calf is born with functioning immune system, but it is immature and not working as sufficiently as in older bovines (Woolums, 2012). This is caused by the protective environment of cow's uterus, for which the newborn calf is immunologically naïve and hasn't had chance to enhance its adaptive immunity by contact with pathogens outside uterus. Due to stress during parturition, calf produces stress hormone cortisol that is known to have immunosuppressive effect on its immune system (Chase *et al.*, 2008). Also, mother's immune system is skewed towards Th2, which is suggested to affect its fetus to have Th2 polarized immunity at birth. This is most likely due to high levels of progesterone and Th2 cytokines produced in maternal-fetal interface (Morein *et al.*, 2002). This predisposes newborn calf susceptible to pathogens. Ingestion of high-quality colostrum quickly after birth helps the newborn calf to adapt to the extrauterine environment, survive and fight against the environment's pathogens (Woolums, 2012). Colostrum provides nutrition for the calf, but most importantly it provides passive immunity in a form of maternal immunoglobulins and other immune components like cytokines, which are suggested to have effect to development and maturation of calf's immature immune system (Gomes *et al.*, 2014).

1.2. Colostrum

1.2.1. Colostrogenesis

Colostrogenesis is a distinct stage of bovine mammary gland development and function, where immunoglobulins from the maternal blood circulation are transferred to the mammary gland secretion in the prepartum period (Barrington *et al.*, 2001). Immunoglobulins begin accumulating selectively by an active receptor-mediated transfer several weeks before parturition from the mother's blood circulation to the mammary secretion through the mammary gland secretory epithelium (Korhonen *et al.*, 2000; Tizard, 2004: 225). Colostrogenesis differs from the production stage of regular milk (lactogenesis) (Barrington *et al*., 2001). Formation of colostrum is thought to be slow and extend to period of four weeks prepartum (Baumrucker *et al*., 2016).

Secretion accumulated in the udder during colostrogenesis is called colostrum, and it is the initial milk secreted from the mammary gland following parturition (Barrington *et al*., 2001; McGrath *et al*., 2016). Colostrum is highly variable in total volume, in the concentration and mass transfer rates of immunoglobulins (Ig), especially IgG_1 (Baumrucker *et al*., 2016). Lactogenic hormones like estrogen, progesterone and prolactin, seems to have an effect to the regulation of colostrogenesis, but there is most likely a local mechanism within the mammary gland also (Barrington *et al*., 2001).

Although, the attention during colostrogenesis is often towards the colostrum's immunoglobulins derived mainly from the mother's blood, we know that some immunoglobulins are produced locally in the udder by plasmacytes that are located close to mammary gland secretory epithelium (Larson *et al*., 1980). Colostrum contains lot of different components in addition to nutrients, of which some are derived from the mother's blood circulation, but also other components that are produced mainly or partly locally in the cow's mammary gland and secreted into the colostrum like cytokines (Hagiwara *et al*., 2000) and acute phase proteins (APPs) like mammary-associated serum amyloid A3 (M-SAA3) (McDonald *et al*., 2001) and haptoglobin (Hp) (Hiss *et al*., 2004).

1.2.2. Composition of colostrum

Colostrum is the initial milk secreted from the mammary gland around the time of parturition (Barrington *et al*., 2001; McGrath *et al*., 2016). Bovine milk can be divided grossly into colostrum and regular milk (Sjaastad *et al*., 2010: 735–760). Colostrum is unique in its function and composition (Barrington *et al*., 2001) and differs from the composition of regular milk (Sjaastad *et al.*, 2010: 735–760). In newborn bovine colostrum provides primarily a complete diet for the calf (Stelwagen *et al.*, 2009; McGrath *et al*., 2016), but colostrum has also important physiological functions (McGrath *et al*., 2016). It is the only source of initial acquired immunity (Stelwagen *et al*., 2009). Furthermore, colostrum ingestion shortly after birth seems to have a role in the maturation of newborn calf's immature immune system (Hagiwara *et al*., 2001).

The energy content of colostrum is doubled, and concentration of protein, fat, vitamins and minerals are higher in colostrum compared to regular milk, but lactose concentration is lower in colostrum (Sjaastad *et al*., 2010: 735–760). Furthermore, colostrum contains immunoglobulins (Korhonen *et al*., 2000; Tizard, 2004: 225–226; Stelwagen *et al*., 2009), complement proteins (Korhonen *et al*., 2000), acute phase proteins (McDonald *et al*., 2001), hormones, cytokines, growth factors, nucleotides, enzymes, polyamines (Blum and Hammon, 2000) and maternal cells like lymphocytes (Woolums, 2012).

Colostrum's composition and physicochemical properties are dynamic and variable (McGrath *et al*., 2016). It contains compounds of cellular and humoral immune defence, which are important for the calf (Korhonen *et al*., 2000). Colostral proteins and maternal cells have effect on the development of calf's functioning immune system. It's known that calves that are fed colostrum containing maternal cells (e.g., lymphocytes) develop the ability to propagate immune response faster compared to calves that were fed colostrum without the maternal cells (Woolums, 2012). Also, some of colostral bioactive agents can modulate digestive and gastrointestinal function (Talukder *et al*., 2002).

Bovine colostrum differs between individuals in its composition (Puppel *et al*., 2009; McGrath *et al*., 2016). Environmental and individual factors affect the composition of the colostrum. These factors are for example parity, pre-partum diet, season, breed, the length

of dry period, vaccination status of the dam, the time of colostrum collection, abortions and the overall health status of the cow (Puppel *et al*., 2019).

1.2.2.1. Immunoglobulins

The plasma cells, which are particular type of antigen-stimulated B lymphocytes, produce antibodies which are also called immunoglobulins (Ig). B lymphocytes are cells of adaptive immune system. Immunoglobulins are antigen-binding receptors, that are released by the cell producing them to the surroundings (Sjaastad *et al*., 2010: 333–354). All immunoglobulins have same kind of basic structure, which molecular weight is approximately 180 kDa (Korhonen *et al*., 2000). Immunoglobulins are glycoproteins (Sjaastad *et al*., 2010: 333–354).

Immunoglobulins' origin in bovine mammary gland secretions are humoral and local (Larson *et al*., 1980; Tizard, 2004: 225–226). Colostrum has higher concentration of immunoglobulins compared to regular milk. Many of them are actively transported by endocytosis from the blood into mammary gland epithelial cells and from the apical membrane by exocytosis to the alveolar lumen. This transport is extremely important in animals, that aren't able to transfer maternal immunoglobulins through placenta to their offspring (Sjaastad *et al*., 2010: 735–760).

The immunoglobulin class and concentration in colostrum and milk varies between species and reflect the origin and route of immunoglobulins (Larson *et al*., 1980). The bovine colostrum is rich in IgG and in lesser extent IgA. There is also IgM and IgE, but in smaller proportions. The major immunoglobulin in bovine colostrum and milk is IgG_1, which accounts 65–90 % of the total antibody content (Tizard, 2004: 225–226). Most of the colostrum IgG_1 is derived from the cow's serum, but some part is suggested to be produced locally (Baumrucker *et al*., 2016). When lactation proceeds further and colostrum turns into milk, less of the antibodies are derived from the cow's serum and more are produced locally in the udder (Tizard, 2004: 225–226).

The cow's serum immunoglobulins possess a wide variety of antibody properties against different antigens to which the cow has been exposed earlier (Larson *et al*., 1980; Woolums,

2012). Calf is born hypo- or agammaglobulinemic, which means it doesn't have significant amounts of immunoglobulins in the blood circulation (Chase *et al.*, 2008). Immunoglobulins in cow's colostrum are known to have protective role to the calf who gets colostrum at a timely manner (Yamanaka *et al*., 2003). Immunoglobulins are critical components of colostrum and ingestion of colostrum at birth provides an immediate source of immunoglobulins for the calf (Chase *et al*., 2008). This is one of resistance factors against infection in newborn calves that is known to affect their immune state (Yamanaka *et al*., 2003).

1.2.2.2. Cytokines

Cytokines are signaling molecules produced by cells of immune system (e.g., neutrophils, macrophages, natural killer, dendritic cells and lymphocytes), that enhance the immune response. Cytokines are group of proteins, regulatory peptides and glycoproteins (Sjaastad *et al*., 2010: 333–354; Reece *et al*., 2011). They are not stored in cells before secretion (Gomes *et al*., 2014). Cytokines are local mediators and regulators, that can work through paracrine or autocrine signalling (Reece *et al*., 2011). They can have endocrine activity as well, which means the cytokines move through blood circulation from one place to another (Tizard, 2004: 134–135). Paracrine means that the cytokines have effect to nearby cells and autocrine means that the released cytokines have effect to the same cell, which produced and released the cytokines. Response to cytokines is seen within seconds (Reece *et al.*, 2011), and their half life varies between the cytokines being five to six days in IL-6 and 20 minutes in TNF-α (Nguyen *et al*., 2007).

Bovine colostrum is rich in cytokines (Hagiwara *et al*., 2000; Tizard, 2004: 226). According to study done by Hagiwara *et al*. (2000) interleukin-1beta (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin-1 receptor antagonist (IL-1ra) were found in bovine colostrum (whey) and in significantly higher concentrations compared to regular milk. The IL-1ra mean concentrations in colostrum were 6 to 32 times higher than IL-1β. IL-1ra has antagonistic effect to IL-1β. All cytokine concentrations decreased in the mammary gland secretion as the lactation period proceeded.

Cytokines are produced in the mammary gland few days before parturition, although the mechanism of cytokine synthesis in the mammary gland isn't fully understood (Hagiwara *et al.*, 2000). Mammary epithelial cells secrete a range of innate immune effector molecules into colostrum and milk, which are white blood cells like neutrophils, macrophages and lymphocytes. These cells in turn secrete immune related components like peptides, antimicrobial proteins (e.g., cathelicidins, lactoferrin and defensins) and earlier mentioned cytokines (Stelwagen *et al*., 2009). Colostrum rapidly improves the ability of neutrophils to phagocytize bacteria, which is primarily accomplished by cytokines (Chase *et al*., 2008). These colostral cytokines may have immunomodulatory effect and influence on the immunity of neonatal calf (Hagiwara *et al*., 2000). It is suggested that the colostral cytokines promote the development and maturation of the immature immune system of the newborn calf (Yamanaka *et al*., 2003; Tizard, 2004: 226).

1.3. Immune development

1.3.1. Innate and adaptive immune system

An innate immune system and an adaptive or acquired immune system are subdivisions, which form the basis of mammalian protective immunity. Both of these subdivisions work together overlapping (Yun *et al*., 2014). The innate immune system mediates early-phase responses to threats and its responses are faster compared to the adaptive immune system (Yates, 2014). After calf's birth the innate immune system doesn't need time for maturation compared to adaptive immune system (Gomes *et al*., 2014).

The innate immunity has two defence lines; the barrier defences such as skin, mucous membranes and secretions, and internal defences such as phagocytic cells, granulocytes, natural killer cells, antimicrobial proteins and inflammatory response (Reece *et al*., 2011). Molecular and cellular mechanisms are used by the innate immune system to fight against potential pathogens. The innate immune system works the same way every time whether or not the pathogen has been encountered earlier (Chase *et al*., 2008; Reece *et al*., 2011), compared to the adaptive immune system, which is pathogen specific, and results in T lymphocyte responses, production of antibodies and memory cells (Yates, 2014).

Development of adaptive immunity and for it to work efficiently, time for maturation of lymphocytes after successive exposures to antigens is needed (Chase *et al*., 2008).

The innate immune system's response is calf's first defence against pathogens (Yun *et al*., 2014). If the barrier defences like mucous membrane in the gastrointestinal tract is breached, the second line of defences in the tissues and blood circulation, which are non-specific against the invader, are activated by the innate immune cells (Reece *et al*., 2011). Macrophages monitor continuously for signs of microbial invasion in the tissues and are important part of an innate immune system (Yates, 2014). Pathogens are recognized through pattern recognition receptors (PRRs), which are expressed on the surface of granulocytes, phagocytes and effector molecules including complements. When macrophages detect microbial products or pathogen-associated molecular patterns (PAMPs) via PRRs, they are activated. This leads to synthesis and release of proinflammatory cytokines (e.g., IL-1β, IL-6, IL-12 and TNF-α) and chemokines (e.g., IL-8) (Yates, 2014; Yun *et al*., 2014). Also, the cells of innate immune system secrete oxidants and lipids (e.g., leukotrienes and prostaglandins) (Tizard, 2004: 17). This process can recruit additional effector cells and molecules to the site to promote the immune response (Yun *et al*., 2014).

Cytokines act as signals for other inflammatory cells and blood vessels in the area. The cytokine release starts a cascade reaction in close by inflammatory cells prompting them to release proinflammatory mediators. The release of cytokines acts directly to blood vessel's endothelium and increases the vascular permeability. This permits the movement of fluid and plasma proteins, such as antibodies, acute phase proteins and complement, into the affected interstitial tissue. Chemokines, which are chemotactic cytokines, can release small particles that set up a concentration gradient in the area of pathogen insult and by this they lure leukocytes, like neutrophils, to the site to fight against pathogens (Yates, 2014).

Besides the local reaction, blood circulation transports cytokines to bone marrow to stimulate the release of stored neutrophils and to promote the production of new neutrophils and monocytes. Neutrophils are usually able to stop mild infections, however if the infection persists the adaptive immune system is activated (Sjaastad *et al*., 2010: 333–354). Dendritic cells work in both innate immune and adaptive immune system, but also innate immune system's macrophages are able to phagocytose and present antigens. They first encounter pathogens and phagocytose them and after this they will move to lymphatic tissue to present

the antigens to naïve B and T lymphocytes to initiate adaptive immune response (Tizard, 2004: 56–66; Kaiser, 2020). The fragment of the invader is presented in the antigen-presenting molecule, which is called MHC molecule, and this structure is moved to the cell surface and presented to lymphocytes. There are two known MHC molecules, MHC I and II, which bind different antigens. This triggers the adaptive immune system (Tizard, 2004: 56).

Adaptive immune response mounted by newborn calf is a primary response, which has longer lag phase and low concentration of antibodies. Newborn tends to produce immune response skewed toward a Th2 rather than Th1 helper cell cytokine pattern (Tizard, 2004: 225). T lymphocytes can be subdivided to $CD4^+$ and $CD8^+$ cells and those cells expressing $CD4^+$ are called T helper cells. These can be further subdivided into cells of Th1 and Th2, which produce their own type of cytokines (Berger, 2000). The placenta produces hormones and cytokines, which have immunosuppressive effect on the cow and the calf by suppressing cell mediated and memory (Th1) responses. These same mediators promote Th2 responses and antibody production (Chase *et al*., 2008). This effects the cellular components, which participate in the innate immune response also (Gomes *et al*., 2014). Most likely this skewing isn't coincidence but essential, because some of the Th1 helper cell cytokines can have detrimental effect during the pregnancy like interferon-gamma (IFN-γ), which is likely to cause damage to the placenta. In healthy adult bovine there is balance between Th1 and Th2 helper cell cytokines toward which the newborn calf's immune responses will progress during the first months of life (Tizard, 2004: 225).

1.3.2. Passive immunity

At the time of birth calf's immune system is functional - although immature. The cells of innate and adaptive immune response don't work like in older animals, which predisposes the newborn calf to infection (Woolums, 2012). At this stage the newborn calf's survival rely heavily on passive immunity acquired via the mother's colostrum, which forms the primary basis for protection against disease (Tizard, 2004: 225; Chase *et al*., 2007; Stelwagen *et al*., 2009). Transfer of antibodies from the mother to the offspring is called passive immunization, which transfers active humoral immunity (Sjaastad *et al*., 2010: 333–354).

The structure of the placenta affects to the route how the fetus or newborn gets its maternal immunoglobulins. Ruminants have syndesmochorial placenta. This type of placenta prevents the transplacental passage of immunoglobulin molecules to the fetus, which means that the newborn calf is totally dependent on immunoglobulins received through the colostrum (Tizard, 2004: 225).

Osaka *et al*. (2014) found out in their study, that newborn calves need to get at least 120 g of immunoglobulins from colostrum preferably in the first hours of their life to acquire adequate passive immunity. While the immunoglobulins are passively transferred to calf's blood circulation where they have a vital role in fight against possible pathogens, the immunoglobulins in colostrum and milk extent the protection against pathogens on the surface of the gastrointestinal tract also (Woolums, 2012). The timely consumption of colostrum is suggested to help intestinal tract colonization by beneficial bacteria, that without would leave the calf vulnerable to possible infections (Fischer *et al*., 2018).

The proteins in the colostrum reach the small intestine of the newborn calf intact. This is because colostrum contains trypsin inhibitors and protease activity of the newborn calf's gastrointestinal tract is low (Tizard, 2004: 226). From there the colostral proteins, including immunoglobulins, are unselectively transferred to calf's blood circulation (Talukder *et al*., 2002; Tizard, 2004: 227). In blood circulation, the maternal immunoglobulins confer protection to specific pathogens to which the cow has been exposed earlier during its life (Woolums, 2012). Colostrum provides an immunologic protection for the calf for the first two to four weeks of life (Chase *et al*., 2008).

The mechanism of passive transfer and intestinal closure aren't known exactly (Fischer *et al*., 2018). Some sources propose that calf's gastrointestinal tract is able to transfer colostral proteins for the first 48 hours after birth (Stelwagen *et al*., 2009; Woolums, 2012). However, in study done by Fischer *et al*. (2018) the calf's intestinal permeability started to decline already after six hours from birth. For utilizing this phenomenon and to get protection against pathogens, it is important for the calf to get enough high-quality colostrum straight after it has been born (Woolums, 2012).

When the calf is few days old, its gastrointestinal tract will start to digest the colostral proteins to smaller amino acids (Woolums, 2012). The amount of passively acquired immunoglobulins start to decline in the calf's serum as the absorption ceases due to normal metabolic processes (Tizard, 2004: 227). The calf's serum immunoglobulin concentration should start to decrease during the first month of its life, which indicates that the calf has had adequate passive immunity. On the other hand, if immunoglobulin concentration increases it usually means that the calf has inappropriate passive protection (Furman-Fratczak *et al*., 2011).

The importance of adequate passive transfer for minimizing morbidity and mortality has been demonstrated (Weaver *et al*., 2000). Calf is considered to have adequate passive immunity, if the measured serum concentration of immunoglobulins is at least 10 g/l or more when measured 30–60 hours after birth (Furman-Fratczak *et al*., 2011). Furman-Fratczak *et al*. (2011) found in their study that calf with adequate passive immunity have the lowest morbidity and intensity of disease course and didn't become ill before the age of 14 days. If the calf's serum immunoglobulin concentration was over 15 g/l, they even avoided respiratory tract infections. Overall, the calves that had acquired adequate passive immunity had better health status and the correct body weight for the first insemination was reached earlier. The main causes of failure of passive transfer (serum Ig concentration <5 g/l) and partial failure of passive transfer (serum Ig concentration <10 g/l) in calves researched by Furman-Fratczak *et al*. (2011) was poor vitality at birth associated with dystocia and low volume of ingested colostrum.

Although colostral immunoglobulins' passive transfer has been traditionally thought to mean the same as passive immunity, it is suggested that there might be more immune factors absorbed from colostrum contributing to the calf's passive immunity and immune development like innate immune proteins, immunomodulatory factors, developmental factors and the presence of cellular immunity (Nissen *et al*., 2017). The biological functions of some of these components and their effect to the active immunity of calf isn't fully known (Gomes *et al*., 2014).

1.3.3. Cytokines' role in immune development

Cytokines are a wide family of soluble signaling polypeptides produced all over the body by almost all cells involved in innate and adaptive immunity (Sjaastad *et al*., 2010: 333–354; Kaiser, 2020). T helper lymphocytes of adaptive immune system are the main cytokine producers (Kaiser, 2020). Cytokines have important functions in the immune defense of newborn calf (Sjaastad *et al*., 2010: 333–354). They regulate innate and adaptive immune system by functioning as chemical messengers mediating the immune response (Sjaastad *et al*., 2010: 333–354; Kaiser, 2020). Cytokines are responsible for many of the biological effects in the immune system, for example cell mediated immunity (Berger, 2000).

The innate immune systems' cells (e.g., macrophages and dendritic cells) produce and secrete variety of cytokines like IL-1, IL-1ra, IL-6, IL-12, IL-18 and TNF-α. Neutrophils have limited ability to secrete cytokines, but at a site of inflammation there are large number of neutrophils, which make together a high number of secreted cytokines at once. Neutrophils secrete IL-1, IL-1ra, TNF-α, IL-6, IL-8, IL-10 and transforming growth factor-beta (TGF-β) (Tizard, 2004: 136–141). Cytokines for example accelerate calf's innate immune response by inducing fever, stimulating the calf's liver to produce and release APPs and recruiting cells of innate immune system like monocytes and neutrophils to the place of inflammation and infection (Sjaastad *et al*., 2010: 333–354). Some cytokines stimulate hematopoiesis (Kaiser, 2020). Cytokines can have adverse effects in form of behavioural signs of sickness (e.g., malaise, lethargy, decrease in appetite) (Tizard, 2004: 42–43).

IL-6 affects many functions in innate and adaptive immunity (Tizard, 2004: 17). IL-6 is able to stimulate the liver hepatocytes to produce APPs during innate immune system's acute phase response. It also stimulates proliferation and differentiation of B lymphocytes and increases neutrophil production. IL-6 is secreted in response to inflammation or trauma by various cells like macrophages, monocytes and T lymphocytes (Kaiser, 2020). Also, bacterial endotoxins, IL-1 and TNF-α are able to stimulate some of the immune system cells (e.g., macrophages and mast cells) to produce IL-6 (Tizard, 2004: 17).

Interferons can modulate the activity of almost every component of immune system. There are type I and type II interferons (Kaiser, 2020). Cytokine IFN-γ is a type II interferon that has a complex, but central role in defending the mammalian host against pathogens. It is

secreted by thymus-derived T lymphocytes and natural killer cells (Boehm *et al*., 1997). Generally, interferons provide early innate immune response against virus infected cells, induce fever and chemokine production to lure leukocytes (Kaiser, 2020). IFN-γ has multiple functions and properties; antiviral agent, regulation of immune response, stimulation of bactericidal activity of phagocytes, stimulation of antigen presentation through MHC class I and II molecules, orchestration of leukocyte-endothelium interactions, effects on cell proliferation and apoptosis, and regulation of over 200 genes (Boehm *et al*., 1997). IFN-γ stimulates macrophages to secrete IL-1, IL-6 and TNF-α, while neutralizing IL-4 actions (Tizard, 2004: 139).

TNF-α mediates acute inflammation, and in excessive amounts it causes severe systemic effects like shock cascade (Kaiser, 2020). It is mainly produced by macrophages and mast cells, but can activate many cells of immune system (e.g., macrophages, mast cells, lymphocytes and neutrophils) (Tizard, 2004: 17). TNF-α stimulates coagulation pathway, chemokine synthesis of macrophages, synthesis of IL-1, liver to produce acute phase proteins and catabolism of fat and muscle for energy. Furthermore, it affects hypothalamus to induce fever and sleep (Kaiser, 2020).

Macrophages are able to produce IL-1, which can be divided into interleukin-1 alpha (IL-1α) and IL-1β. From these two IL-1β is secreted while IL-1α is attached to the macrophage and works when in direct contact with target cell (Tizard, 2004: 17). IL-1 mediates acute inflammation and works synergistically with TNF-α to enhance inflammation (Kaiser, 2020). IL-1β is suggested to significantly increase number of neutrophils in blood circulation, O_2^- production in neutrophils and induce band cell migration from bone marrow (Hagiwara *et al*., 2001). IL-1ra is suggested to reduce the adverse side-effects of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) and contribute to the immunological maturation of neonates (Hagiwara *et al*., 2000).

T helper lymphocytes can be distinguished by the cytokines they secrete to Th1 and Th2 helper cells. Th1 helper cells secrete cytokines for example interleukin-2 (IL-2), IFN-γ and TNF-α, while Th2 helper cells secrete cytokines like interleukin-4 (IL-4), interleukin-10 (IL-10), interleukin-13 (IL-13) and TNF-α. These cells are activated by antigen and costimulators, which are presented by different antigen-presenting cells. Myeloid dendritic

cells and B cells with the help of costimulators (e.g., specific molecule and IL-12) present antigens to Th1 helper cells inducing their response. They promote cell-mediated immune responses (e.g., macrophage activation and delayed hypersensitivity reaction). Th1 helper cells lack IL-1 receptor, which means that in the absence of IL-12 the T helper cell response switches from Th1 helper cell response to Th2 helper cell response. Th2 helper cells respond mainly to antigen presented by lymphoid or plasmacytoid dendritic cells and macrophages, but not that well to B cell antigen presentation. Th2 helper cell response is induced by costimulatory molecule and cytokine IL-4, but IL-1 from macrophages and dendritic cells may also be required. Th2 helper cell cytokines stimulate B cell proliferation and immunoglobulin production and secretion (e.g., IgG_1, IgA and IgE), while having no effect on cell-mediated reactions (Tizard, 2004: 113–114).

Th1 and Th2 helper cell cytokines have activities that counteract the activities of the other one. Hence, the balance between these subsets of Th helper cells determines the immune response to a specific antigen. Principal functions of cytokines secreted by Th1 helper cell are IL-2 that causes activation of T cells, B cells, natural killer cells and macrophages and IFN-γ that causes inhibition of Th2 cells, stimulation of Th1 cells, activation of natural killer cells and macrophages. The functions of cytokines secreted by Th2 helper cells are for example IL-4 that stimulates B cell growth and differentiation and activation of mast cells and IL-10 that inhibits Th1 cell function and suppresses function of macrophages (Tizard, 2004: 138–139).

1.4. Acute phase response

1.4.1. Cytokines' role in acute phase response initiation

The disturbance of calf's homeostasis by tissue trauma, infection, immunological disorder or neoplasia, propagates the acute phase response (APR) (Heinrich *et al*., 1990). Innate immune system function is amplified during APR, while adaptive immune reactions are suppressed (Berczi *et al*., 2009). APR is part of defence mechanisms of innate immunity due to acute inflammation or infection, which leads to release of APPs (eClinPath; Kaiser, 2020). Proinflammatory cytokines are the inducers of the APR and the head mediator is IL-6

(Tizard, 2004: 43–44). APPs measured, for example from serum, represent the cytokine and innate immune system activity.

When activated cells of innate immune system like macrophages and dendritic cells PRRs come in contact with PAMPs, they release inflammatory cytokines (Kaiser, 2020). Main cytokines initiating this defence response are IL-1, TNF-α and especially IL-6 (Tizard, 2004: 43–44; Yates, 2014). These cytokines travel through the blood circulation to liver where they stimulate hepatocytes to increase the synthesis and secretion of APPs (Tizard, 2004: 43–44; Kaiser, 2020). Also, it is shown that there are other organs like gastrointestinal tract, kidneys, heart, mammary gland and adipocytes that are able to synthesize various APPs (Yates, 2014). The concentration of APPs starts to increase already in few hours due to the cytokine stimulation and quite often subsides in 24–48 hours (Tizard, 2004: 43).

1.4.2. Acute phase proteins in bovine

APPs are a group of serum proteins, which concentration changes in response to acute inflammation or infection (Heinrich *et al*., 1990). This change is stimulated mainly by cytokines IL-1, IL-6 and TNF-α, which trigger liver to produce and secrete APPs (Hiss *et al*., 2004). Acute phase proteins that can be found from bovine blood are for example serum amyloid A (SAA), haptoglobin (Hp), lipopolysaccharide binding protein, fibrinogen, alpha-1-acid glycoprotein and lactoferrin (Yun *et al*., 2014). In dairy cows the serum concentration of Hp increases in the first week of postpartum as does SAA (Johns, 2015).

There are several known APPs, but in ruminants the major APPs are SAA and Hp. The major APP is one whose concentration increases even 100 to 1000 folds during APR (eClinPath). Traditionally, APPs are used as markers of inflammation or infection and measuring them from blood is used as aid in assessing health in general, the severity of infection and the efficacy of treatment given (Tothova *et al*., 2014). The formation of APPs due to cytokine activity indicates the functioning of innate immune system.

SAA has been found in all studied vertebrates and it is evolutionarily strongly conserved (Eckhardt *et al*., 2010). It is predominantly synthesized in liver hepatocytes due to cytokine stimulation during APR (Larson *et al*., 2005). There are at least four different known gene

variants of serum amyloid A found from mammals; SAA1, SAA2, SAA3 and SAA4 (Olsen *et al*., 2013; De Buck *et al*., 2016). In bovine gene variants SAA1 and SAA2 are suggested to be synthesized in acute situations while high concentrations of SAA3 is produced in mammary gland at the time of parturition (Larson *et al*., 2005). Larson *et al*. (2005) demonstrated in their study that prolactin stimulates production and secretion of bovine mammary-associated serum amyloid A3 (M-SAA3) in mammary epithelial cells. At the same time, it didn't stimulate the production of acute serum amyloid A (acute-SAA) in detectable amounts. Therefore, the authors suggest that gene that encodes M-SAA3 is independently regulated from the other genes that encode the production of acute-SAA1 and acute-SAA2.

The immunologic function of the acute-SAA is to recruit leukocytes chemotactically (Yates, 2014; Johns, 2015). This means it attracts neutrophils, T lymphocytes and monocytes (Tizard, 2004: 44). Acute-SAA is suggested to be able to regulate immune responses, because of its immunosuppressive effect – it downregulates inflammatory response (Tizard, 2004: 44; Yates, 2014). It has an inhibitory effect on fever and oxidative burst of neutrophils. Acute-SAA is involved in lipid metabolism and transport also (Yates, 2014). Acute-SAA may have concentration dependent effects. Furthermore, it takes part in regulation of innate and adaptive immune responses (De Buck *et al*., 2016).

De Buck *et al*. (2016) reviewed the regulated expression of acute-SAA in normal and transformed cells and compared its serum levels in various disease states in humans. Their analysis revealed that acute-SAA acts as chemotactic and induces cytokines in very low tissue concentrations (early inflammation; 10–100 ng/ml). When compared to acute-SAA concentrations of 1000 ng/ml or more in ongoing infection, where liver produces continuously more and more acute-SAA, this acute phase protein is working directly as bacterial opsonin and interfering with virus infection. Also, it has anti-inflammatory effect to host in high concentrations. This means, that in high acute-SAA concentrations (10 µg/ml or more) it tries to protect the host from the immune reactions damage by working through negative feedback mechanism.

The concentration of haptoglobin (Hp) increases, because of the activation of transcription due to the APR (Johns, 2015). Hp can be produced in liver and non-hepatic places of inflammation like mammary gland (Hiss *et al*., 2004; Huntoon *et al.*, 2008). Hp inhibits

granulocyte chemotaxis and phagocytosis (Yates, 2014). It binds hemoglobin and sequesters iron from microbes, which inhibits bacterial proliferation and invasion (Tizard, 2004: 44; Yates, 2014). Haptoglobin has effect to development and differentiation of the organs of immune system (Huntoon *et al*., 2008). Its concentration in calf can be in normal situation undetectably low and rapidly increase for example in case of respiratory disease (Tizard, 2004: 44). Furthermore, it may regulate and modulate the immune response at the time of inflammation (Johns, 2015). Haptoglobin induced by inflammation helps to accomplish an ideal immune response (Huntoon *et al*., 2008).

Colostrum contains acute phase proteins like mammary-associated serum amyloid A3 (M-SAA3), which is one of the four known isoforms of SAA, and haptoglobin (Hp) (McDonald *et al*., 2001; Hiss *et al*., 2004; Thomas *et al*., 2016). Traditionally, acute phase proteins are measured from blood and used as markers of inflammation or infection (Tizard, 2004: 43–44). During the time of parturition, it seems that increased concentrations of M-SAA3 and Hp in colostrum are part of normal nonpathological process (McDonald *et al*., 2001; Thomas *et al*., 2016). Maternal stress induced by parturition seems to extend its effect to the mammary gland in a way that colostrum contains these APPs (Thomas *et al*., 2016). Larson *et al*. (2005) demonstrated in their study that prolactin stimulates production and secretion of bovine M-SAA3 in mammary epithelial cells. The M-SAA3 and Hp concentrations in the mammary gland secretion start to decrease by the fourth day postpartum (McDonald *et al*., 2001; Thomas *et al*., 2016).

McDonald *et al.* (2001) found in their study M-SAA3 isoform to be abundant in the colostrum of healthy bovine, ovine and equine and at lower levels in milk. The mature M-SAA3 is a 113 amino acid protein (apolipoprotein) with molecular mass of 12.8 kDa, that is secreted by bovine mammary gland epithelial cells. Expression of M-SAA3 seems to be normal part of physiologic process that isn't only limited with disease associated responses. The authors speculate that M-SAA3 may have important and beneficial role in the well-being of the newborn and in the maintenance of the mammary gland. Larson *et al*. (2005) suggest that M-SAA3 in colostrum has a vital role in establishing a healthy gastrointestinal tract for a newborn calf by inducing mucin production, which can hinder the pathogens' ability to adhere to the gastrointestinal wall and cause disease.

1.5. Possible role of colostral cytokines, mammary-associated serum amyloid A3 and haptoglobin to calf's adaption to extrauterine life

Alsemgeest *et al.* (1995) studied two major bovine acute-phase proteins; SAA and Hp concentrations in plasma of newborn calves. They concluded that a fetal calf doesn't form APPs due to prenatal stress during parturition when they compared different delivery methods, which were spontaneous parturition, normal or heavy extraction and cesarean section. In many of the diseased calves the plasma Hp concentration remained low although their SAA concentration was significantly higher compared to the healthy newborn calves. This indicates that newborn calves are able to produce SAA. In adult bovines both SAA and Hp rise almost simultaneously. Due to this difference, Alsemgeest *et al*. (1995) suggests that newborn calves aren't able to produce Hp, because their hepatic synthesis isn't fully developed yet, or it takes longer time to be produced.

Orro *et al.* (2006) studied the temporal changes in SAA and Hp concentrations and their associations with weight gain in healthy newborn reindeer calves. They found that during the first month of life both SAA and Hp had age-related changes in the serum concentration. Serum SAA concentration increased significantly between the first and second week of life. Hp concentrations increased during the whole observation period. Authors state that these time-related changes of APPs have a role in the adaptation and defence mechanisms of newborn reindeer calves, but the reasons for these changes are unknown. They suggest that possible reasons might be the ingestion of colostrum containing mediators of the APR, age-related changes in hepatic synthesis of APPs and the newborn's exposure to pathogens. The results of the study suggested that colostrum influences the hepatic synthesis of APPs, especially SAA, in reindeer calves.

Temporal changes in serum concentration of four different APPs in newborn dairy calves have been studied by Orro *et al.* (2008). They studied SAA, lipopolysaccharide binding protein, α1-acid glycoprotein and Hp. Also, colostral samples and serum samples for SAA was studied to find out, if the colostral SAA is passively transferred to calf. Furthermore, SAA isoforms in calves were identified and compared to SAA isoforms identified in colostrum. Authors found that there were temporal changes of all the studied APPs during the first three weeks. They also found out that colostral SAA isoforms weren't the isoforms found from calves' serum, which indicates that calves are able to form SAA themselves.

Authors suggest that colostrum intake causes the changes in serum concentrations of APPs and especially the colostral inflammatory mediators, like cytokines, may stimulate calf's liver to produce APPs. The results from the study suggest that birth process and its possible traumas to the calf and/or some colostral stimulatory factors are associated with the temporal changes of APPs in newborn dairy calves.

Mack *et al*. (2003) studied the possible regulatory effect of acute phase protein M-SAA3 to the small intestine MUC3 mucin expression. They found in the study that M-SAA3 can alter the mucin expression in small intestine by increasing the mucin production. This layer of mucin may prevent the pathogens access to the underlying intestinal epithelial cells. For the newborn animal this is extremely important in the time when their adaptive immune system is relatively ineffective. Colostrum contains bioactive peptides like M-SAA3, which ability to modulate the expression of small intestinal mucins may be important mechanism in enhancing the innate immune system's ability to fight against enteric pathogens while newborn is adapting to extrauterine life.

Hiss-Pesch *et al*. (2011) investigated the transmission of maternal Hp to suckling piglets. They found out that maternal Hp is transmitted from the sow via colostrum to the newborn suckling piglets. After three hours from colostrum intake, serum Hp concentration in newborn piglets increased compared to the colostrum deprived newborn piglets where the serum Hp concentration stayed low. Furthermore, they observed that the ingestion of colostrum stimulated the endogenous Hp synthesis in newborn piglets. The synthesis of Hp in the liver increased after nine hours from birth in the piglets that ingested colostrum, whereas in the piglets deprived of colostrum didn't show increase of Hp mRNA in the first 12 hours of life. This suggest that the piglets' Hp synthesis is stimulated by colostrum.

Arredouani *et al.* (2003) found in their research that Hp has a balancing effect in immune responses. Hp has direct inhibitory and anti-proliferative effects on T lymphocytes. T helper cell type 1 (Th1) and T helper cell type 2 (Th2) are responsible for induction and regulation of humoral and cellular responses of adaptive immunity. Furthermore, the effect of haptoglobin on Th1 release is only slightly inhibitory (IL-2) or absent (IFN-γ), but strongly inhibitory effect is seen in release of Th2 cytokines (e.g., IL-4, IL-5, IL-10 and IL-13), which mediated responses are predominantly humoral and eosinophilic. Therefore, Hp promotes

Th1 activation over Th2 activation. This suggests that Hp has a modulating role in the Th1-Th2 balance and promotes Th1 cellular response.

Huntoon *et al*. (2008) generated Hp deficient mice to study the effect of Hp on the function of immune cells. They discovered that this APP has the ability to regulate host immunity. The Hp deficient mice had undeveloped lymphoid organs (smaller spleen, lymph nodes and thymus) and due to this decreased amount of mature B and T lymphocytes in the body. The T lymphocyte relative composition, when compared to normal mice, where the number of $CD8^+$ (T cytotoxic cells) and $CD4^+$ (T helper cells) cells were in balance, was now shifted to favour the $CD8^+$ cells. The fewer number of lymphocytes due to Hp deficiency suggests that the proliferation, activation and differentiation of lymphocytes is weakened in these newborn mice. Furthermore, the adaptive immune system responses in mice were substantially reduced. Authors conclude that Hp expression by hematopoietic cells is needed for optimal immune response. Also, Hp seems to act as one of the coactivators of T lymphocytes between the APR and immune response. The biggest impact of Hp seems to be in the regulation of lymphocyte growth in homeostasis and after antigen stimulation.

Hagiwara *et al*. (2000) studied bovine colostrum and milk whey concentrations of cytokines (IL-1β, IL-6, TNF-α, IFN-γ and IL-1ra) with immunomodulatory properties. Also, cytokine (IL-1β, IL-6, TNF-α, IFN-γ) mRNA expression in cells from colostrum and mature milk was examined. They detected studied cytokines in all colostrum samples (days 0–5) and in some mature milk samples. They found that concentrations of IL-1β, IL-6 and TNF-α were highest on the day of parturition in colostrum compared to other stages of lactation, which was divided to early, mid and later stages. Concentration of IFN-γ was significantly higher at the day of parturition and on day three in colostrum compared to milk in late stage of lactation. On the early or mid stages of lactation, IFN-γ was not detected. IL-1ra concentration in colostrum was significantly higher compared to mature milk. Additionally, authors found a positive correlation between the colostrum concentrations of IL-1ra and IL-1β. The concentration of IL-1ra in colostral whey was found to be 6–32 times higher compared to IL-1β, and at the time of parturition the concentration of antagonist was six times higher and after five days postpartum the concentration was 32 times higher compared to IL-1β. This suggests that the IL-1ra reduces the adverse side-effects of proinflammatory cytokines IL-1β, IL-6 and TNF-α and contributes to the immunological maturation of

neonates. Authors suggest that IL-1ra inhibitory effect through oral administration needs further investigation. All of the studied cytokine mRNAs were observed in colostral cells on the day of parturition and in low concentrations in cells of mature milk throughout the lactation period. Exception was observed on the fifth day when only cytokine IL-1β expression was observed. Author suggests that this may indicate that colostral cytokines are produced in the mammary gland during the last few days before giving birth to calf, although the mechanism of cytokine synthesis is still unclear.

Colostral cytokine IL-1β has been demonstrated by Hagiwara *et al*. (2001) to be able to move through passive transfer after administering it via oral route to the newborn calves after birth. Also, Hagiwara *et al.* (2001) studied its immunological effects on the neonatal calves. The IL-1β was detected in the blood circulation after an hour from the administration, from where the concentration level increased for one more hour. After six hours from the administration, the IL-1β wasn't detected in the blood anymore. Furthermore, white blood cell count, mainly neutrophils, increased after 12 hours in all calves that received IL-1β. The number of band neutrophils in blood circulation were increased 12 hours post-administration, indicating that this cytokine might induce migration of band cells from bone marrow. Its effect to numbers of neutrophils in blood circulation was seen until 48 hours from birth although the IL-1β is known to be undetectable at this point. This may suggest that IL-1β may stimulate other cytokines like IL-6 and TNF-α, which have their own effects. This cytokine significantly increased proliferation of peripheral blood mononuclear cells that were stimulated with concavalin A, and the stimulant mediated production of O_2^- in neutrophils increased significantly after 6–12 hours of the administration of the IL-1β. Hagiwara *et al.* (2001) demonstrated that stimulation with LPS, even after colostrum feeding, had no effect on the proliferation of peripheral blood mononuclear cells in newborn calves. Due to this, the authors suggest that the passive transfer of IL-1β via oral route effects the proliferation of T lymphocytes, but not B lymphocytes. That is why it is suggested that oral administration of IL-1β has an immunostimulatory activity in the newborn calves. Authors suggest that colostral cytokines and their inhibitors may contribute to appropriate immune system maturation of newborn calf.

In study done by Yamanaka *et al.* (2003) proinflammatory cytokines were found from serum of calves that had been fed colostrum much earlier compared to colostrum deprived calves.

At the time of parturition, the concentration of cytokines (IL-1β, IL-6, TNF-α, IFN-γ and IL-1ra) were undetectable low in the serum, but at 12 hours of age in the colostrum fed calves the cytokine concentrations were increased to detectable levels and peak level was reached in 24 hours. After this, the serum cytokine concentrations decreased until four weeks of age after which they weren't detectable anymore. In colostrum deprived calves, cytokines (IL-1β, IL-6, TNF-α, IFN-γ and IL-1ra) were detected in serum three days after birth. Author suggests that the origin of the calves' serum cytokines is colostrum, although cytokine mRNA expressions have been found from the newborn calves' peripheral blood mononuclear cells without link to the concentration level of cytokines in the calves' serum.

Gomes *et al.* (2014) studied the influence of bovine colostrum intake and development of innate immune response of calves from birth to two months of age. Calves were negative of all studied proinflammatory cytokines (IL-1β, IL-6, TNF-α, IFN-γ) before the ingestion of colostrum at birth. They found that calf's colostrum intake influences the serum IFN-γ concentration and the proportions of monocytes ($CD14^+$) and neutrophils ($CH138^+$) in the blood circulation. Authors suggest that these immune components may have an important part in the enhancement of calf's cellular immune response and protection against pathogens by enhancing the phagocytic activity and antigen presentation by monocytes.

1.6. Conclusions of literature review

The importance of colostrum ingestion for the newborn calf's survival and adaptation to extrauterine life is generally acknowledged. Cow's colostrum is known to confer passive immunity to the calf in form of maternal immunoglobulins, but studies indicate that there may be more colostral components absorbed through passive transfer like colostral cytokines, which have important effects to the calf's immune system development and maturation. Newborn calves are negative for all proinflammatory cytokines before ingestion of colostrum. It is proved that colostral cytokines are passively transferred from colostrum. Also, acute phase proteins like Hp may be passively transferred, but it seems that SAA or at least M-SAA3 isn't. Nevertheless, both of these APPs are suggested to have effect to the calf's immune system development. Hp is suggested to contribute to immune organ development and when induced by inflammation enables propagation of proper immune response. Also, Hp helps to achieve normal homeostatic balance between Th1 and Th2

helper cells, which is known to be skewed at birth. Colostral M-SAA3 has protective role in the gastrointestinal tract by inducing mucin production in small intestine, which improves the innate immune system's barrier defences. Colostral cytokines, especially IL-6, IL-1 and TNF-α, are suggested to stimulate the newborn calf's liver to produce acute phase proteins like acute-SAA and Hp. Studies suggest that colostral cytokines and their inhibitors may have role in the maturation and modulation of the immune system in neonates. Colostral cytokines may stimulate the innate immune system's functions by enhancing innate immune cells' phagocytic activity and monocytes antigen presentation, activating neutrophils and T lymphocytes, stimulating the proliferation of T lymphocytes and band neutrophils migration from the bone marrow. Although, few studies suggest colostral cytokines to have effect on the development of calf's functioning immune system, more research is needed to get broader understanding of the possible effects of colostrum's active components. By studying colostral cytokines associations to newborn calves' serum APPs concentrations, one may find out if these colostral cytokines may effect the calves' immune response development.

2. AIM OF THE STUDY

Aim of the study is to investigate possible associations of colostral cytokines (IL-1β, IL-6) and acute phase proteins (SAA and Hp) to the same variables in calves' serum in a research period from birth until three weeks of age after one-time ingestion of colostrum after birth.

3. MATERIALS AND METHODS

3.1. Study material

This current study uses a part of a research material from a large-scale study done by Niine *et al*. (2018), which described and studied the *Cryptosporidium parvum* outbreak in female calves and the effect of treatment with halofuginone lactate and the association with inflammatory response and short-term weight gain. The study was conducted in a large dairy farm situated in Järvamaa County, which is in Central-Estonia. At the time of study material gathering, there were approximately 1,800 dairy cows in the herd. For this study, calves' serum samples from the first three weeks of life and samples of the colostrum fed to calves were used. The interest was on concentrations of the interleukin-1β (IL-1 β), interleukin-6 (IL-6), serum amyloid A (SAA) and haptoglobin (Hp) in colostrum and especially in calves' serum samples from birth until three weeks of age when they had been fed colostrum once after birth. Next, a description of study material used for this study is given, but for more detailed description of the overall research material can be found from the study Niine *et al.* (2018).

All the female calves born during the period starting from 21st of January to 16th of March 2015 were included in this study. The cows that were chosen to the study were either primiparous or multiparous. Number of calves in the study population was 144. Twin calves and male calves were excluded.

Immediately after birth calves were separated from their mothers and put into individual pens, where they were kept the entire study period. Within the first two hours of life calves were fed three litres of unpasteurised colostrum milked from their mother. Before feeding the colostrum, its quality was assessed visually and with a hydrometer (Kruuse colostrum densimeter, Jørge Kruuse A/S, Langeskov, Denmark). If colostrum quality was insufficient, calf was fed with earlier deep-frozen good quality colostrum from another dam. There were two cases where the dam's colostrum wasn't good quality, and deep-frozen colostrum was used for the calves. Then up to 15–17 days of age calves were fed twice a day 2–3 kg warmed unpasteurised raw milk. Same time calves had free access to starter feed (Prestarter,

Agrovarustus OÜ, Tartu, Estonia) and hay. After this, calves' unpasteurised raw milk was switched to milk powder (Josera GoldenSpezial, Josera GmbH & Co. KG, Kleinheubach, Germany). Three kilograms of this milk powder solution (1 litre of warm water mixed with 140 g of milk powder) was given to calves two times a day for a week. During this period free access to hay and starter feed continued.

Parainfluenza virus type 3 and bovine respiratory syncytial virus vaccine (Rispoval, Zoetis Belgium SA, Louvain-la-Neuve, Belgium) was given to every calf at two days of age. Also, prophylactic treatment of *Cryptosporidium* was done with halofuginone lactate (Halocur, Intervet International B.V., Boxmeer, Netherlands) to about 70% of calves born. First group of calves didn't get treatment, but second group got treatment when they were over two days of age lasting under a week (incorrectly treated) and third group got treatment within two days of age lasting at least a week or longer (correctly treated). All the animals in the study were treated by the farm's veterinarian, if needed.

3.2. Sample collection

Serum samples were collected once a week from each calf. Generally, there were one to three serum samples taken from each calf during the entire study. Each sample was taken from the calf's jugular vein using an 18G sterile needle and a sterile evacuated test tube. In the laboratory, the samples were centrifugated (4,500 rpm for 10 min) after which the serum samples were stored at -20 °C before further analysis was done.

Colostrum sample was taken at a time before the first feeding of the colostrum to each calf. The colostrum sample was collected into sterile 10 ml vial. After this the sample was stored in aliquots at -20 °C before further analysis. Colostrum samples were centrifugated (10,000 rpm for 10 min) and the fat layer was removed before the laboratory analyses.

Furthermore, fecal samples were collected from the calves to study the possible *Cryptosporidium* infection. All together one to three fecal samples were taken from each calf during the study period, but there was one calf that wasn't sampled at all. The fecal sample was collected directly from the rectum of the calf with disposable latex glove. A clean and selable plastic cup was used to retain the sample before its analysis. The collected

fecal samples were kept at temperature of +4 °C before further analysis of possible protozoa. The maximum storage period for fecal samples from the collection of sample to analysis was 48 hours.

Total number of serum samples obtained from 144 calves during this study period from the first three weeks of calves' life was 383. From all of the serum samples obtained, 126 serum samples were taken during the first week of life (age 1 to 7 days old calves). Total of 133 serum samples were taken in the second week of life (age 8 to 14 days old calves) and the rest 124 serum samples were taken during the third week of life (age 15 to 21 days old calves). The number of calves sampled was the same as the number of serum samples in that week. There were 328 fecal samples taken from the study population of calves. From the total number of fecal samples, 103 samples were taken during the first week of life, 112 samples were taken during the second week of life and 113 samples were taken during the third week of life. There was a total of 144 colostrum samples taken just before the feeding.

3.3. Laboratory analysis

Colostrum and serum samples' cytokine concentrations were determined by using a commercial bovine IL-1β and IL-6 ELISA kits (Cusabio Biotech, Wuhan, Hubei, China) as instructed by the manufacturer. Detection limit for IL-1β was 15.6 ng/l and for IL-6 it was 2.5 ng/l. Another commercial ELISA kit (Phase BE kit, Tridelta Development Ltd., Dublin, Ireland) was used to measure the serum and colostrum samples' SAA concentrations, which detection limit for this specific acute phase protein was 0.3 mg/l. Colostrum immunoglobulin G (IgG) concentration was determined by commercial ELISA kit (BIO K 165/2 kit, Bio-X Diagnostics S.A., Rochefort, Belgium). Haptoglobin concentration from serum and colostrum samples was measured using a method described by Makimura and Suzuki (1982) with the exception of using tetramethylbenzine (60.0 mg/l) as a substrate and microtitration plates (Alsemgeest *et al.*, 1994). The method's detection limit for Hp was 60.0 mg/l.

An immunofluorecence method was used to detect approximate *Cryptosporidium* oocysts count in fecal samples modified by Niine *et al.* (2018). Fluorescein isothiocyanate (FITC) conjugated anti-*Cryptosporidium* monoclonal antibodies (Crypto Cel, Cellabs Pty Ltd., Sydney, Australia) were used for sample staining. Calves were grouped into three depending

on the number of oocysts detected in the fecal sample during each study week (no oocysts detected – negative, detected number of oocysts below the median value – low oocysts level, and number of detected oocysts above the median value – high oocysts level), which was used in the statistical analysis.

3.4. Statistical analysis

Multiple linear regression models were used for investigating possible associations of colostrum and serum inflammatory markers concentrations by study week. Logarithmic transformations of APR markers (SAA, Hp, IL-6) serum concentrations by study week were used as response variables. Tobit regression models were used for serum IL-1β (logarithmically transformed). Because >60% of the serum samples IL-1β concentrations were under the detection limit of the assay (15.6 ng/l) the Tobit regression model was chosen. In the Tobit regression, all cases falling above (or below) a specified threshold value are censored, although these cases remain in the analysis (Long, 1997). Colostrum SAA, Hp, IL-1β, IL-6 and IgG concentrations were included as covariates. All models initially included age at sampling (days) and time from birth to the colostrum ingestion (minutes) as covariates; and halofuginone lactate treatment group (no treatment, incorrectly treated, correctly treated), *Cryptosporidium* oocyst level group (no oocysts, under the median oocysts count at the sampling week and over the median oocyst count) at the time of sample, mother parity and aid at parturition (no or yes) as categorical explanatory variables. The final models were produced by backward elimination of the variables from the initial models. Interactions and confounders (change of coefficient over 10% after variable elimination) were controlled.

Assumptions of final models were controlled using normality and scatter plots of model residuals and $p \leq 0.05$ was considered statistically significant. Analyses were performed using Stata/IC 14.2 (StataCorp LP, College Station, TX, USA).

4. RESULTS

The measured concentrations of components in the colostrum fed to the calves in the study regarding the studied cytokines (IL-1β and IL-6) and acute phase proteins (Hp and SAA) are presented in table 1. Also, colostrum IgG concentration is presented. There were 144 colostrum samples analysed.

Table 1. Mean and median concentrations of the studied colostrum components

Colostrum component	Statistic	Mean and median concentrations (n = 144)
Interleukin-1β (ng/l)	Mean (±SD)	547.6 (±1134.8)
	Median (min-max)	115.1 (15.6–5445.1)
Interleukin-6 (ng/l)	Mean (±SD)	55.5 (±53.2)
	Median (min-max)	43.5 (10.2–501.4)
Haptoglobin (mg/l)	Mean (±SD)	189.9 (±61.6)
	Median (min-max)	174.6 (103.7–479.5)
Serum amyloid A (mg/l)	Mean (±SD)	66.0 (±47.0)
	Median (min-max)	52.2 (7.5–277.0)
Immunoglobulin G (g/l)	Mean (±SD)	55.1 (±11.9)
	Median (min-max)	54.2 (26.8–104.5)

Note. Symbol "n" means the number of colostrum samples used in the calculations.

The concentrations of interest (IL-1β, IL-6, Hp and SAA) measured in calves' serum during the first three weeks of life is presented in table 2. Number of serum samples were the same as number of calves sampled in the age group.

Table 2. Mean and median concentrations of calves' serum variables (IL-1β, IL-6, Hp and SAA) during the first three weeks of life (age from 1-21 days old calves grouped by age of sampling to one, two and three weeks of life)

Calves' serum variables	Statistic	First week of life (n = 126)	Second week of life (n = 133)	Third week of life (n = 124)
Interleukin-1β (ng/l)	Mean (±SD)	99.1 (±227.9)	23.8 (±32.6)	49.0 (±55.6)
	Median (min-max)	15.6 (15.6–1321.5)	15.6 (15.6–207.4)	33.3 (15.6–444.8)
Interleukin-6 (ng/l)	Mean (±SD)	16.0 (±18.9)	10.2 (±14.2)	10.4 (±8.4)
	Median (min-max)	9.5 (0.1–117.0)	5.9 (0–130.7)	8.6 (0.1–47.5)
Haptoglobin (mg/l)	Mean (±SD)	368.8 (±432.9)	694.3 (±655.2)	564.5 (±537.7)
	Median (min-max)	193.0 (97.0–2662.0)	406.0 (95.0–2830.0)	192.0 (85.0–3310.0)
Serum amyloid A (mg/l)	Mean (±SD)	143.7 (±66.6)	140.4 (±75.2)	92.4 (±61.4)
	Median (min-max)	128.8 (22.4–347.7)	125.2 (34.0–487.9)	78.5 (13.2–371.9)

Note. Symbol "n" means the number of serum samples taken from calves and used in the calculations.

The two studied serum cytokine (IL-1β and IL-6) concentrations acted similarly in the age groups from one week to three weeks of age (Table 2). Serum mean concentration was highest during the first week of life, then during the second week of life the concentration decreased and during the third week of life it increased again. Serum acute phase protein concentrations (Hp and SAA) acted differently compared to each other (Table 2). The mean serum Hp concentration increased from the first week to the second week of life, but during the third week serum Hp concentration decreased, but not as low as it was during the first week of life. On the contrary compared to Hp, the mean serum SAA concentration was highest during the first week of life and decreased gradually until the third week of life. In all the studied serum variables the mean concentration was higher than median concentration, which means that the distribution was skewed positively and wasn't normally distributed.

Box and whisker plots were used to describe the data collected from the study population. The figures show the calves' serum average IL-1β (Figure 1), IL-6 (Figure 2), Hp (Figure 3) and SAA (Figure 4) concentrations and their distribution (e.g., median, min, max, 1st quartile (25%) and 3rd quartile (75%)) in the study population arranged to age by week. There were three age groups: first, second and third week of life. The 1st week of life group consisted serum samples from calves aged 1 to 7 days old (n = 126). The 2nd week of life group consisted serum samples from calves aged 8 to 14 days old (n = 133) and last 3rd week of life group was from 15 to 21 days old (n = 124).

In table 2 and in figure 1 the results concerning the serum IL-1β concentrations are presented. Figure 1 illustrates the measured concentrations during the three first weeks of calves' life. The mean serum IL-1β concentration was highest during the first week of life (mean ± SD; 99.1 ± 227.9 ng/l). During the second week of life mean serum concentration decreased (23.8 ± 32.6 ng/l) compared to the concentration level during the first week of life and increased slightly during the third week of life (49.0 ± 55.6 ng/l) from the concentration level of the second week. The median concentration was during the first and second week of life on the threshold of the detection limit but increased during the third week (Table 2). Furthermore, figure 1 shows the distribution of the measured concentrations in the study population, and a positive association ($p < 0.001$) between colostrum IL-1β concentration and calves' serum IL-1β concentration (n = 103; 70 left-censored and 33 uncensored) was observed during the first week of life. No association was observed later during second or third week of calves' life between serum IL-1β concentration and any of the studied colostrum components (Hp, SAA, IL-1β or IL-6).

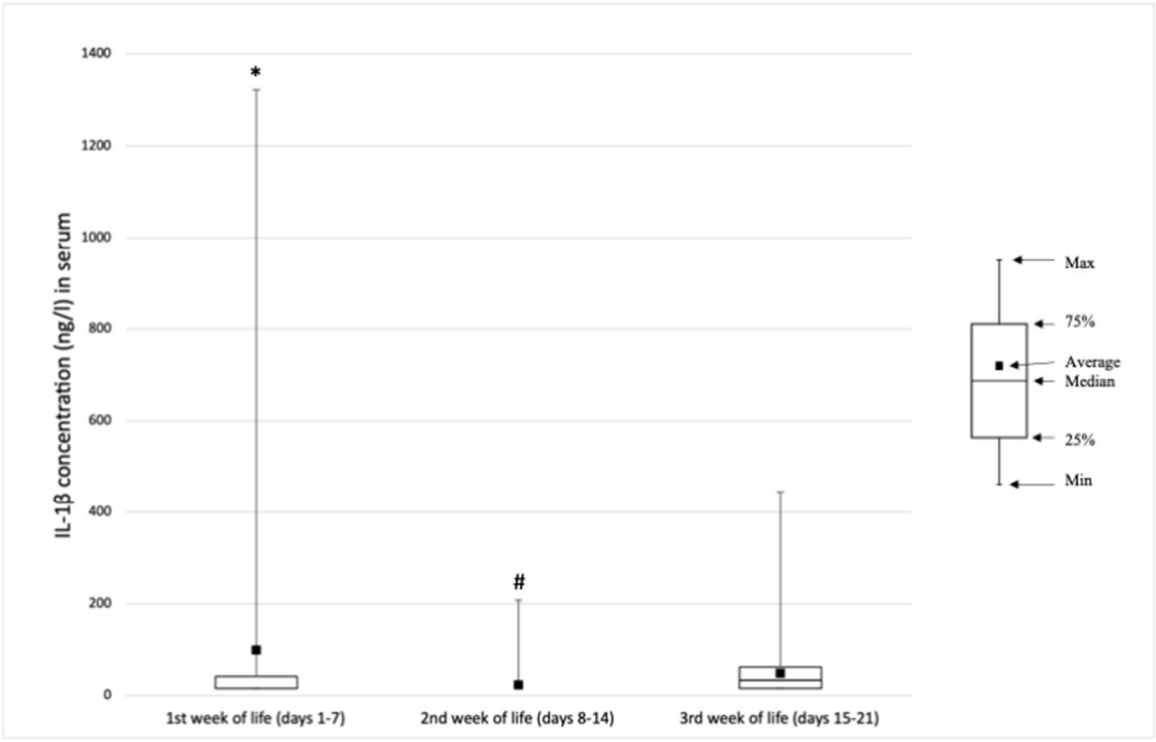

Figure 1. Cytokine interleukin-1β (IL-1β) concentration (ng/l) in calves' serum during the first (n = 126), second (n = 133) and third week of life (n = 124).
* Significant positive association with colostrum IL-1β concentration ($p < 0.001$) evaluated by Tobit regression model after logarithmic transformation of serum IL-1β values.
# The used assay wasn't sensitive enough for detection of low concentrations of IL-1β from serum samples.

The mean serum IL-6 concentration was highest during the first week of life (16.0 ± 18.9 ng/l). In the second week of life the concentration decreased (10.2 ± 14.2 ng/l) and then minimally increased from the level in the second week during third week of life (10.4 ± 8.4 ng/l). This and study population's serum concentration of IL-6 distribution between the age groups is illustrated in figure 2. During the first week of calves' life there was a positive association ($p < 0.001$) observed between colostrum IL-6 concentration and calves' serum IL-6 concentration (n = 103). Similarly, there was a positive association observed during the second week of life (n = 112) and during the third week of life (n = 124) between colostrum IL-6 concentration and calves' serum IL-6 concentration (2^{nd} week $p < 0.001$ and 3^{rd} week $p = 0.001$). Other associations between calves' serum IL-6 concentration in the age groups and colostrum components weren't found.

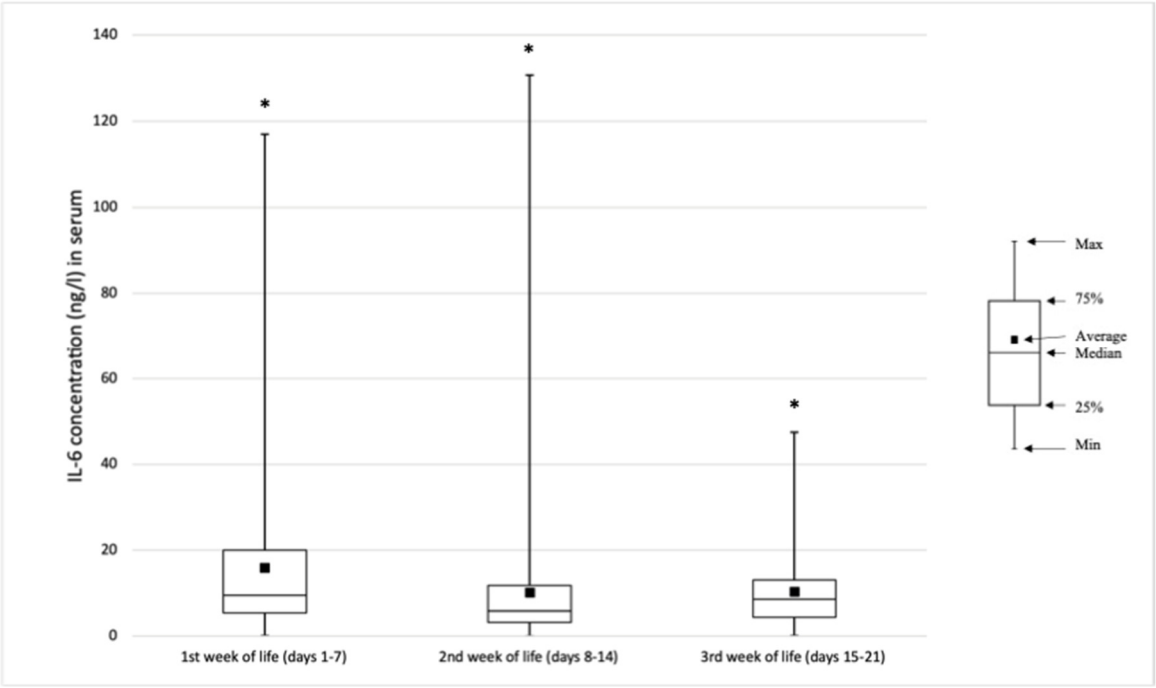

Figure 2. Cytokine interleukin-6 (IL-6) concentration (ng/l) in calves' serum during the first (n = 126), second (n = 133) and third week of life (n = 124).
* Significant positive association with colostrum IL-6 concentration (1st week $p < 0.001$, 2nd week $p < 0.001$ and 3rd week $p = 0.001$) evaluated by linear regression model after logarithmic transformation of serum IL-6 values.

In figure 3 calves' serum haptoglobin (Hp) concentration in the age groups is illustrated. The mean Hp concentration in serum was the lowest during the first week (368.8 ± 432.9 mg/l) and increased to its highest level during the second week of life (694.3 ± 655.2 mg/l) from where the concentration decreased during the third week of life (564.5 ± 537.7 mg/l) but stayed somewhat higher than in the first week of life. In the second week of life the interquartile range (the middle 50% of study population's values) was largest. The distribution of measured serum Hp concentrations in study population are found from table 2. During the first week of calves' life there was a positive association ($p < 0.001$) observed between calves' serum Hp concentration and colostrum IL-6 concentration (n = 102). There were no associations observed between calves' serum Hp concentration and any of the studied components (IL-1β, IL-6, Hp and SAA) in colostrum in the second and third week of life. Also, there were no associations found between colostrum Hp concentration and any of the serum variables studied.

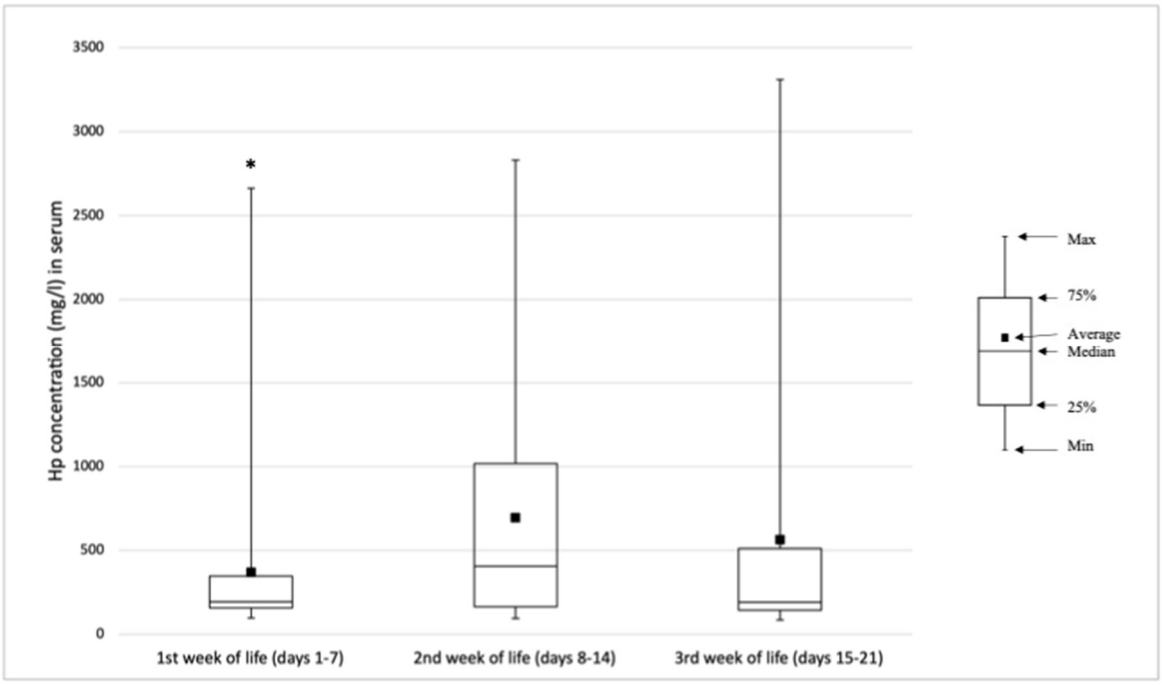

Figure 3. Haptoglobin (Hp) concentration (mg/l) in calves' serum during the first (n = 126), second (n = 133) and third week of life (n = 124).
* Significant positive association with colostrum IL-6 concentration ($p < 0.001$) evaluated by linear regression model after logarithmic transformation of serum Hp values.

Serum amyloid A (SAA) mean concentration in calves' serum was in its highest during the first week of life (143.7 ± 66.6 mg/l). From there on, it gradually decreased during the next two weeks (2nd week 140.4 ± 75.2 mg/l and 3rd week 92.4 ± 61.4 mg/l). This is demonstrated in figure 4. The study population's serum SAA concentration distribution is presented in table 2. There were no observations of association between calves' serum SAA concentration and any colostrum components (IL-6, IL-1β, SAA and Hp) during the first three weeks of calves' life (age 1 to 21 days old). Similarly, no association was observed between colostrum SAA and any of the studied serum variables.

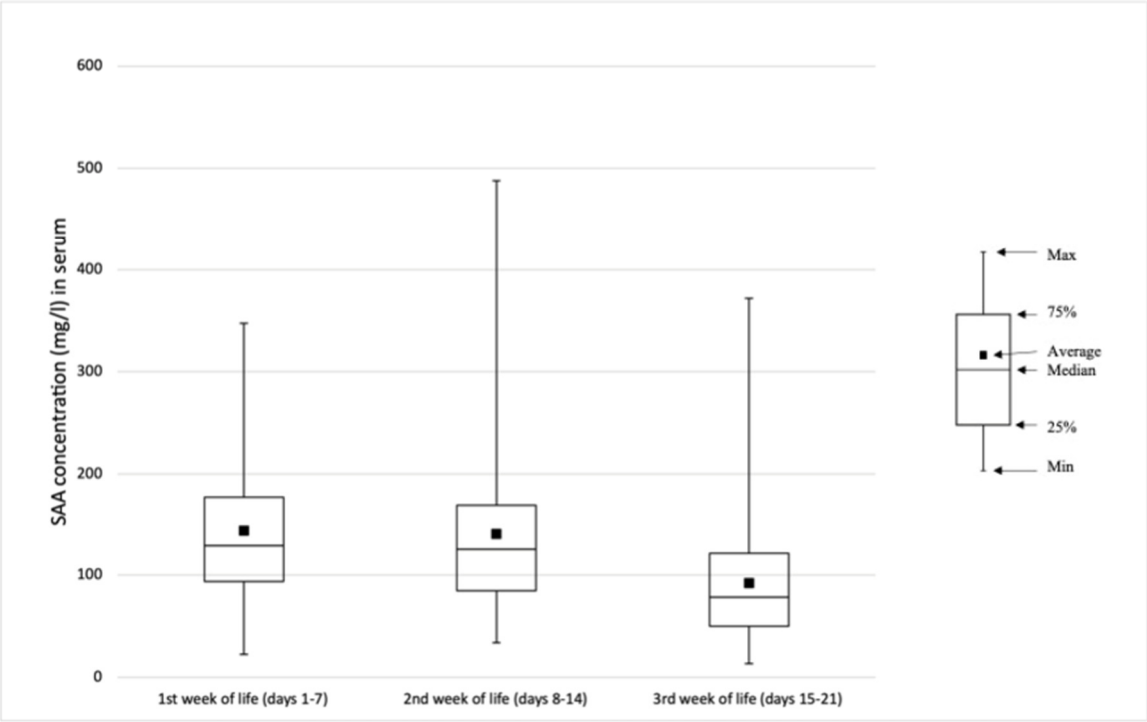

Figure 4. Serum amyloid A (SAA) concentration (mg/l) in calves' serum during the first (n = 126), second (n = 133) and third week of life (n = 124).

5. DISCUSSION

The results of mean and median concentrations of studied cytokines (IL-1β and IL-6) and APPs (Hp and SAA) in the bovine colostrum fed to calves in this study (Table 1) has remarkably lower concentrations of IL-1β and IL-6 compared to research done by Hagiwara *et al.* (2000) and higher concentrations of acute phase proteins Hp and SAA when compared to study by Thomas *et al*. (2016). In study by Orro *et al.* (2008) mean colostrum SAA concentration was closer to the mean value measured in this study. Previous studies of newborn calves and their serum cytokine concentrations have shown that calves are negative of cytokines when born and it takes longer for cytokines to be detected in their serum, if they are colostrum deprived (Yamanaka *et al*., 2003; Gomes *et al*., 2014). If they ingest colostrum soon after birth, the cytokines are detected in the serum earlier (Yamanaka *et al.*, 2003). In this study there were no serum sampling of calves done before the colostrum feeding, which means that the finding of cytokine negative calves at birth can't be supported by this study. Also, there were no comparison group of calves that didn't get colostrum because of ethical reasons. The differences in the colostrum IL-1β, IL-6, Hp and SAA concentrations compared to other studies may be explained by the differences in used methods, in study populations (e.g., size of study population) and individual animals that are known to affect the cow's colostrum composition in general (e.g., number of calvings) (Puppel *et al.*, 2009; McGrath *et al.*, 2016).

In this study mean serum IL-1β and IL-6 concentrations were highest during the first week of life from where they decreased during the second week of life and slightly increased again in third week of life (Figure 1; Figure 2; Table 2). Similar results have been published earlier with IL-1β (Yamanaka *et al.*, 2003). Possible reason for the trend observed during the first and second week of life in the study may be that ingested colostrum cytokines increase the serum cytokine concentration in calf during the first week and during the second week the serum cytokine concentration decreases because of half-life. The cytokine half-life for example IL-6 is known to be five to six days (Nguyen *et al.*, 2007). Also, calves weren't sampled considering the possible half-lives of different studied variables, which may have effect to the results. The mild increase during the third week of life may be because of calves' own cytokine synthesis is starting to work and the maternal immunity starts to fade gradually

in the second to fourth week of life (Chase *et al.*, 2008), which challenges the calves' immune system to work.

The colostrum cytokine IL-1β concentration was observed to have a positive association with the same variable in calves' serum during the first week of life in this study, which may be caused by passive transfer of this cytokine from colostrum and/or it may stimulate calf's cytokine synthesis. This observation of association is supported by the findings in earlier research done by Hagiwara *et al*. (2001). In the second and third week of life associations weren't observed. The assay that was used to measure the serum IL-1β concentration wasn't sensitive enough to detect the low concentrations of this cytokine accurately (detection limit was 15.6 ng/l) from the serum (Figure 1). Because of this, one can't rely on the results that there is or isn't association observed between serum IL-1β and any other colostrum variables studied during the second week of age. The used assay gave a concentration result of the detection limit to all the measurements that were undetectable. Over 60% of the measured serum concentrations were under the detection limit and for this reason a Tobit model was used in statistical evaluation of this studied cytokine concentration.

This study revealed colostrum IL-6 concentration having a positive association with the calves' serum IL-6 concentration in the entire study period from first week to three weeks of age. These results suggest that colostral IL-6 may affect directly to calves' serum IL-6 concentration during the first week of life, for which one may assume that colostrum ingestion soon after birth and the passive transfer of colostrum IL-6 is the most likely reason for this association, like observed with IL-1β, or/and colostrum IL-6 stimulates the calf's own cytokine synthesis. The positive association between colostrum and serum IL-6 concentrations during the second and third week of calves' life can't be directly caused by single colostrum ingestion at birth when considering the cytokine half-life (Nguyen *et al*., 2007) and the time when passive transfer from the gastrointestinal tract is possible (Stelwagen *et al*., 2009; Woolums, 2012; Fischer *et al.*, 2018). It seems that there may be indirect long-term effect of colostrum IL-6 causing the association during the second and third week of age. This association might be due to colostrum's maternal cells (e.g., lymphocytes) transferred by passive transfer to calves' blood circulation (Stelwagen *et al*., 2009; Woolums, 2012; Gomes *et al*., 2014) while these cells are still stimulable and able to produce and secrete cytokine IL-6 in the calves' blood circulation for a longer period.

However, the real cause of this association isn't known yet and needs further scientific research.

Haptoglobin mean and median concentration in calves' serum was observed to increase from the first week to the second week of life from where the serum Hp concentration started to decrease during the third week of life, which has been observed to act similarly in a study by Orro *et al*. (2008). The colostrum's Hp concentration was not observed to be associated with calves' serum Hp concentration from birth to three weeks of age, but colostrum cytokine IL-6 concentration was observed to have a positive association to calves' serum Hp concentration during the first week of life. Any associations between serum Hp concentration and colostrum SAA or IL-1β weren't observed. Cytokine IL-6 is a known mediator of acute phase response and it is known to be able to stimulate liver to produce APPs like Hp and SAA (Hiss *et al.*, 2004).

Previous study done in newborn piglets suggested that colostrum Hp is absorbed passively from the intestines to the blood circulation and may cause increase in piglets' serum Hp concentration, because the serum Hp concentration in the piglets increased after ingestion of colostrum (Hiss-Pesch *et al.*, 2011). The results from this study indicate no association between colostrum Hp and calves' serum Hp concentrations during the study period. Based on this finding, the lack of an association between colostrum and calves' serum Hp concentrations may indicate that colostrum Hp isn't either absorbed by passive transfer from calf's intestine to the blood circulation or Hp is absorbed in insufficient concentration to have effect to calf's serum Hp concentration.

Haptoglobin is said to be able to stimulate its own production in cells (Hiss-Pesch *et al.*, 2011) and to be needed for the development, maturation, and modulation of newborn calf's immune system and its optimal response (Arredouani *et al*., 2003; Huntoon *et al.*, 2008). However, it is suggested in earlier study that calf's Hp production is slow or that hepatic synthesis isn't fully developed at the time of birth to produce Hp but needs time for the physiological maturation to be able to produce Hp (Alsemgeest *et al*., 1995). The result of this study challenges the possible theories of passive transfer of colostrum Hp and its association to newborn calf's serum Hp concentration. However, the result of this study provides a new insight into the relationship between colostrum IL-6 and calves' serum Hp. Colostrum IL-6 and calves' serum Hp concentrations were observed to have a positive

association with each other during the first week of life. Possible interpretation may be that passively transferred colostral IL-6 stimulates calf's liver to produce Hp causing the observed increase of Hp concentration in the calves' serum in the first week of life. This IL-6 immune system stimulation from colostrum may be extremely important for the calf's survival. It may hasten the newborn calf's immune system maturation and adaptation to the new environment, because in previous study by Yamanaka *et al*. (2003) showed that cytokines are detected in calf's serum only after three days from birth when calf is colostrum deprived. In colostrum ingested calf serum cytokines were detected within the same day when born (Yamanaka *et al*., 2003). Also, Hp has known effects to the immune system. It promotes Th1 activation (cellular response) over Th2 activation (Arredouani *et al*., 2003), which means that in newborn calf, whose immune system is Th2 balanced when born, it has modulating role and helps to shift the skewed Th1-Th2 balance to the centre. The results of this study suggest that colostrum cytokine IL-6 may have a role in development of calf's immune system response and survival with the help of other colostrum components that confer passive immunity (e.g., immunoglobulins).

Previous study in calves and colostral APPs have shown that colostrum SAA components aren't the same isoforms that are found from calf's serum (Orro *et al.*, 2008). It has been observed in earlier study that colostrum is rich in M-SAA3 and it seems that this APP isn't passively transferred to calf's serum but conveys its important effect in the gastrointestinal tract by aiding in calf's first line barrier defences (Mack *et al*., 2003). The results of this study build on to this existing evidence of SAA not transferring from colostrum at all or at least not in sufficient concentration to calf's blood circulation. Earlier studies have suggested that calves are able to produce SAA after they are born (Alsemgeest *et al*., 1995; Orro *et al*., 2008), which is supported by the findings of this study. In the study there were no associations observed between colostrum and serum SAA concentration, or with any of the studied colostrum components and calves' serum SAA concentration. The concentration level of SAA in calves' serum was highest during the first week of life from where it gradually decreased until the end of the third week of life. This similar serum SAA concentration pattern has been observed in study done by Orro *et al.* (2008). One may form a hypothesis that there may be some other cytokine in colostrum, which has a positive effect on the serum concentration of SAA after ingestion of colostrum or even synergistical effect of colostral cytokines that weren't studied. It is known that colostrum contains multiple

different cytokines and other active components, which true effects are mostly still uncovered and can only be speculated (Gomes *et al*., 2014; Nissen *et al*., 2017).

6. CONCLUSIONS

Aim of this study was to investigate possible associations between IL-1β, IL-6, Hp, SAA concentrations in bovine colostrum and calves' serum. The study revealed significant positive associations between colostrum cytokine IL-1β and IL-6 concentrations and calves' serum IL-1β, IL-6 and Hp concentrations within study period. Results suggest that studied colostrum cytokines may be transferred to calves' serum and/or stimulate the calves' own cytokine production. Associations between colostrum APPs (SAA and Hp) and studied serum variants (IL-1β, IL-6, Hp, SAA) in calves, weren't observed in the study. Colostrum IL-6 was observed to have a positive association to calves' serum IL-6 concentrations in all three age groups of calves. Furthermore, colostrum IL-6 had a positive association to calves' serum Hp only during the first week of life. Colostrum cytokine IL-1β was observed to have a positive association with calves' serum IL-1β during the first week of life. Any other IL-1β associations weren't observed, which may be affected by the sensitivity of used assay.

Results of the study suggest that studied colostrum cytokines may stimulate calf's naïve immune system response and development to fully functioning, while the colostrum's maternal antibodies are aiding in the calf's survival. It may be that due to colostrum cytokine stimulation the calf's immune system is matured until the maternal antibodies are faded significantly. Further scientific work is needed with cytokine IL-1β to find out precisely, if there is or isn't associations between colostrum components (IL-1β, IL-6, Hp, SAA) and serum IL-1β during the first three weeks of life. More sensitive assay is needed to get more accurate measurements from the calves' serum IL-1β concentrations. Also, investigating and finding the possible cause for the colostrum IL-6 long-term association to calves' serum IL-6 concentration in the second and third week of life needs further research. Other colostrum cytokines, their effects and associations to calves' immune system maturation, modulation and development may be of interest in the future.

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APPENDIXES

Appendix 1. Non-exclusive licence for depositing the final thesis and opening it for the public and the supervisor's (supervisors') confirmation for allowing the thesis for the defence

Hereby I, **Anna-Leena Honkala**
09.01.1985

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ERRATA

The data of calves' serum cytokine interleukin-6 (IL-6) concentrations from the first three weeks of calves' life contained some incorrect values that were measured to be lower than the used assay's detection limit of 2.5 ng/l. The data was corrected, and calves' serum IL-6 concentrations were recalculated for the first three weeks of life, which are presented in the corrected Table 2. The minimum value was 2.5 ng/l after correction. Because of the error in the data, Figure 2 was corrected based on the recalculations.

Table 2. on page 35.

Table 2. Mean and median concentrations of calves' serum variables (IL-1β, IL-6, Hp and SAA) during the first three weeks of life (age from 1-21 days old calves grouped by age of sampling to one, two and three weeks of life)

Calves' serum variables	**Statistic**	**First week of life (n = 126)**	**Second week of life (n = 133)**	**Third week of life (n = 124)**
Interleukin-1β (ng/l)	Mean (±SD)	99.1 (±227.9)	23.8 (±32.6)	49.0 (±55.6)
	Median (min-max)	15.6 (15.6–1321.5)	15.6 (15.6–207.4)	33.3 (15.6–444.8)
Interleukin-6 (ng/l)	Mean (±SD)	16.1 (±18.8)	10.4 (±14.1)	10.5 (±8.2)
	Median (min-max)	9.50 (2.5–117.0)	5.9 (2.5–130.7)	8.6 (2.5–47.5)
Haptoglobin (mg/l)	Mean (±SD)	368.8 (±432.9)	694.3 (±655.2)	564.5 (±537.7)
	Median (min-max)	193.0 (97.0–2662.0)	406.0 (95.0–2830.0)	192.0 (85.0–3310.0)
Serum amyloid A (mg/l)	Mean (±SD)	143.7 (±66.6)	140.4 (±75.2)	92.4 (±61.4)
	Median (min-max)	128.8 (22.4–347.7)	125.2 (34.0–487.9)	78.5 (13.2–371.9)

Figure 2. on page 38.

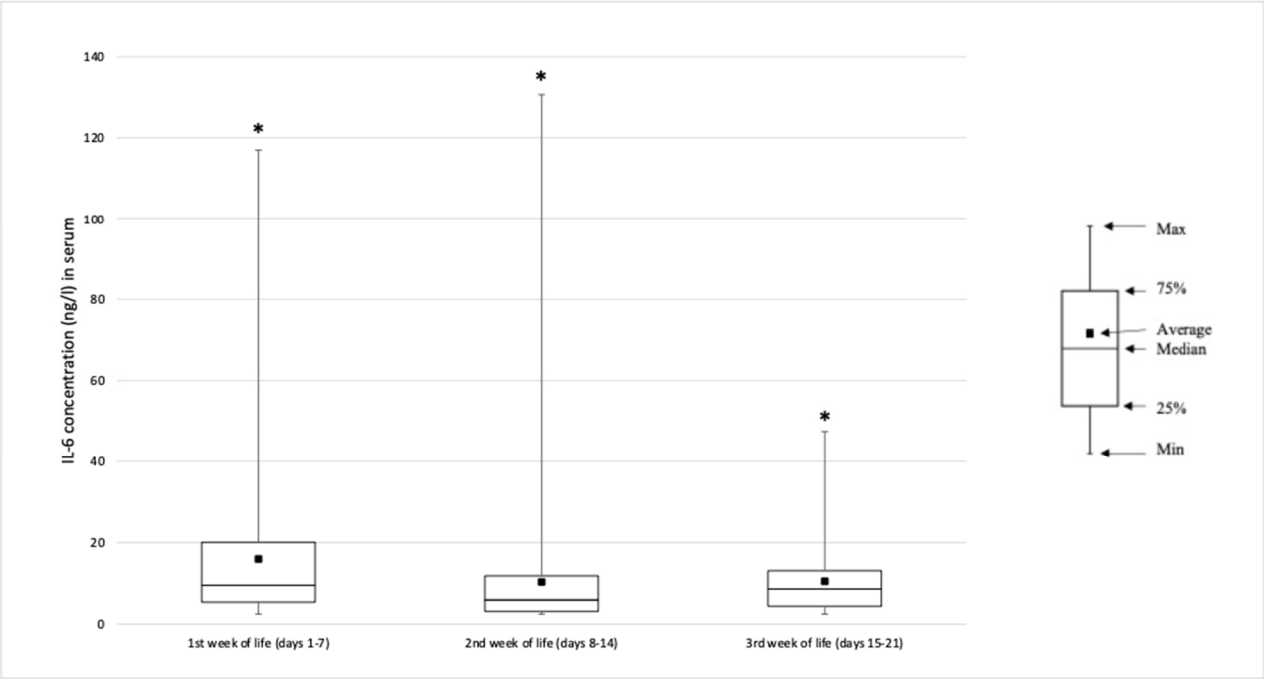

Figure 2. Cytokine interleukin-6 (IL-6) concentration (ng/l) in calves' serum during the first (n = 126), second (n = 133) and third week of life (n = 124).

* Significant positive association with colostrum IL-6 concentration (1st week $p < 0.001$, 2nd week $p < 0.001$ and 3rd week $p = 0.001$) evaluated by linear regression model after logarithmic transformation of serum IL-6 values.